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Ribosomal protein synthesis is not regulated at the translational level in *Saccharomyces cerevisiae*: balanced accumulation of ribosomal proteins L16 and rp59 is mediated by turnover of excess protein

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We have investigated the mechanisms whereby equimolar quantities of ribosomal proteins accumulate and assemble into ribosomes of the yeast *Saccharomyces cerevisiae*. Extra copies of the *cry1* or *RPL16* genes encoding ribosomal proteins rp59 or L16 were introduced into yeast by transformation. Excess *cry1* or *RPL16* mRNA accumulated in polyribosomes in these cells and was translated at wild-type rates into rp59 or L16 proteins. These excess proteins were degraded until their levels reached those of other ribosomal proteins. Identical results were obtained when the transcription of *RPL16A* was rapidly induced using *GAL1–RPL16A* promoter fusions, including a construct in which the entire *RPL16A* 5′-noncoding region was replaced with the *GAL1* leader sequence. Our results indicate that posttranscriptional expression of the *cry1* and *RPL16* genes is regulated by turnover of excess proteins rather than autogenous regulation of mRNA splicing or translation. The turnover of excess rp59 or L16 is not affected directly by mutations that inactivate vacuolar hydrolases.

[*Key Words*: Yeast ribosome biosynthesis; assembly-mediated turnover]

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A significant fraction of macromolecular synthesis in yeast cells is devoted to the production of ribosomes. The approximately 75 different ribosomal proteins comprise 15% of the total mass of yeast protein and the 4 ribosomal RNA molecules represent 85% of the mass of yeast RNA. Ribosomal proteins are synthesized in the cytoplasm and enter the nucleolus rapidly where they co-assemble with ribosomal RNA precursors into ribosomal subunits. The process appears to be very efficient in that equimolar quantities of each ribosomal protein and RNA accumulate; only a negligible excess of any unassembled molecule is detectable (reviewed in Warner et al. 1986). The balanced accumulation of ribosomal components occurs not only during steady-state growth but also when the rate of ribosome synthesis is altered in response to changes in physiological conditions (Kief and Warner 1981; Kim and Warner 1983a).

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We would like to determine the mechanisms that govern this efficient synthesis and assembly of yeast ribosomes. Ribosomal proteins and ribosomal RNAs appear to be synthesized in roughly equimolar amounts (Udem and Warner 1972; Gorenstein and Warner 1976; Kim and Warner 1983b). The presence of conserved promoter sequences upstream from most yeast ribosomal protein genes suggests that ribosomal protein synthesis may be regulated in part at the level of transcription (Rotenberg and Woolford 1986; Woudt et al. 1986; Larkin et al. 1987; Schwindinger and Warner 1987). In fact, the coordinate increase in ribosomal protein synthesis during a nutritional upshift and the decrease upon heat shock result from changes in rates of transcription of ribosomal protein genes (Kim and Warner 1983a; Donovan and Pearson 1986; Herruer et al. 1987).

Artificially induced imbalances in gene dosages have been used to show that coordinate synthesis of ribosomal proteins in *Escherichia coli* is coupled to ribosome assembly by a translational feedback mechanism (reviewed in Nomura et al. 1984). The translation of each polycistronic ribosomal protein mRNA is inhibited by a ribosomal protein encoded in that operon when that

protein accumulates in excess of assembling ribosomes. Introduction of extra copies of individual cistrons encoding these repressor ribosomal proteins results in an inhibition of the translation of the polycistronic mRNA transcribed from the regulated operon.

Transformation or injection of extra copies of ribosomal protein genes or mRNAs into eukaryotic cells has provided evidence for posttranscriptional regulation of ribosomal protein production at the level of pre-mRNA processing, mRNA stability, translation, and protein turnover (Pearson et al. 1982; Bozzoni et al. 1984; Himmelfarb et al. 1984; Abovich et al. 1985; Warner et al. 1985; Dabeva et al. 1986; El Baradi et al. 1986; Bowman 1987; Baum et al. 1988; Maicas et al. 1988; Pierandrei-Amaldi et al. 1988; M. Jacobs-Lorena, pers. comm.). We have used this approach to investigate which if any of these posttranscriptional processes modulates the expression of the yeast *cry1* and *RPL16* genes encoding ribosomal proteins rp59 or L16. We found that excess rp59 or L16 proteins are synthesized in proportion to excess mRNAs present in the cell, and are degraded until they reach levels identical to other ribosomal proteins. We obtained no evidence for autogenous regulation of splicing, stability, or translation of these mRNAs.

Results

Increased abundance of cry1 *and* RPLI6 *mRNAs in strains with elevated dosage of the* cry1 *and* RPL16A *genes*

Derivatives of the high copy number plasmid YEp24 that contain the ribosomal protein genes *RPL16A* or *RPL16B* (Woolford et al. 1979) or *cry1* (Larkin and Woolford 1983), were isolated from a yeast genomic library (Carlson and Botstein 1982) or constructed from previously cloned fragments. These plasmids were transformed into yeast strain RL78 to obtain cells with elevated dosage of the *RPL16* or *cry1* genes. Each of these plasmid borne ribosomal protein genes is expressed in yeast. The *RPL16A* or *RPL16B* plasmids complement *rpl16* null mutations (Rotenberg et al. 1988). Cells of genotype *CRY1* are sensitive to cryptopleurine (Cry^S). When those cells are transformed with the high copy plasmid YEp24cry1 they become Cry^R due to an excess of the *cry1* gene product expressed from the plasmid (Larkin and Woolford 1983). Because the copy number of these plasmids varied among different transformants, we screened a number of independent transformants for those that contained the highest copy number of each DNA. Total DNA was isolated from transformed cells, digested with a restriction endonuclease that linearized the plasmid DNA, subjected to gel electrophoresis, and assayed by Southern blot analysis using a ^{32}P-labeled *CRY1* or *RPL16A* probe. The copy number of each plasmid was estimated by counting radioactivity in the chromosomal and plasmid bands containing the *CRY1*, *cry1*, or *RPL16A* genes. Most of the transformants contained from 15 to 20 molecules of plasmids YEp24cry1 or YEp24L16A per cell (Fig. 1). Thus, there was a 7- to 10-fold increase in the dosage of the *RPL16* and *CRY* genes in their respective transformants, as there are two copies of both of these genes per haploid yeast genome (Rotenberg et al. 1988; A.G. Paulovich et al., in prep.).

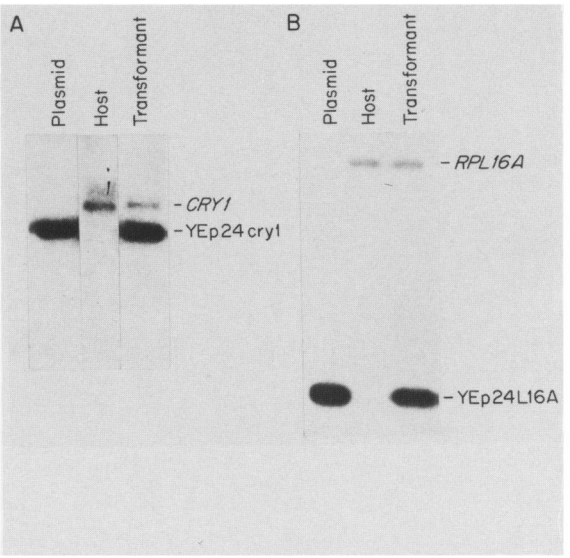

Figure 1. Elevated dosage of *cry1* or *RPL16A* genes in cells transfromed with YEp24cry1 or YEp24L16A, respectively. DNA was extracted from yeast strain RL78 and from RL78 transformants, digested with *Bam*HI (*A*) or *Bam*HI plus *Sal*I (*B*), subjected to electrophoresis, transferred to nitrocellulose, and hybridized with *A* ^{32}P-labeled 2.2-kb *CRY1 Hin*dIII fragment or *B* 1.1-kb *RPL16A Bgl*II–*Bam*HI fragment. The genomic and plasmid DNA fragments homologous to each probe are indicated. (*A*) Determination of copy number of YEp24cry1 transformed into RL78. (*First lane*) 10 ng of YEp24cry1 plasmid DNA; (*second lane*) 1 μg of untransformed RL78 genomic DNA; (*third lane*) 1 μg of DNA from RL78 YEp24cry1 transformant. (*B*) Determination of copy number of YEp24L16A transformed into RL78. (*First lane*) 10 ng of YEp24L16A plasmid DNA; (*second lane*) untransformed RL78 genomic DNA; (*third lane*) DNA from RL78 YEp24L16A transformant.

RNA gel transfer analysis demonstrated that an approximate 8- to 10-fold excess of *cry1* mRNA accumulated in the YEp24cry1-transformed strains and a 5- to 15-fold excess of *RPL16* mRNA accumulated in YEp24L16-transformed strains, compared to the YEp24 vector transformants (Fig. 2A, B). In each case, the ribosomal protein mRNAs were quantitated by densitometry or scintillation counting, relative to a yeast mRNA homologous to plasmid pY11-10 (Woolford and Rosbash 1979) or to yeast *ACT1* mRNA (Gallwitz and Seidel 1980), to control for differential loading of RNA on each gel lane. Because the excess of *cry1* or *RPL16* mRNA in the transformed cells was equal to the net increase in dosage of the respective genes, we conclude that transcription or stability of these mRNAs is not modulated to a significant extent in response to increased gene dosage.

Expression of several ribosomal protein genes in frogs and yeast is regulated at the level of pre-mRNA processing (Bozzoni et al. 1984; Dabeva et al. 1986). However, we found no evidence for regulation of the splicing of the intron-containing *cry1* pre-mRNA. Although an

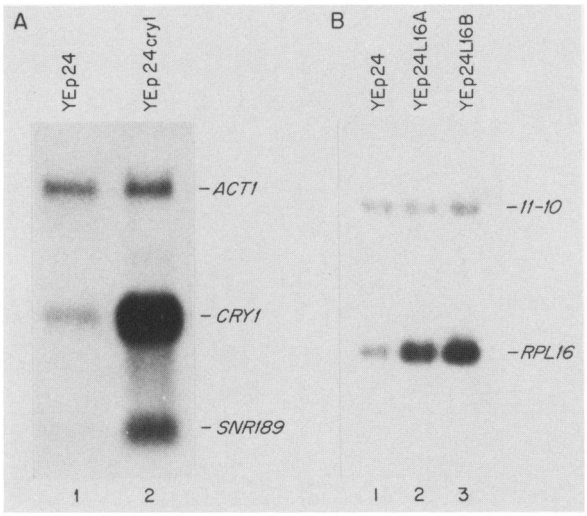

Figure 2. Increased abundance of *cry1* or *RPL16* mRNAs in cells transformed with YEp24cry1, YEp24L16A, or YEp24L16B. RNA was extracted from RL78 yeast transformed with YEp24 (*A* and *B*, lane *1*) and YEp24*cry1* (*A*, lane *2*), YEp24L16A (*B*, lane *2*), or YEp24L16B (*B*, lane *3*). The RNA was fractionated by electrophoresis on 1.2% agarose denaturing gels, transferred to nitrocellulose, and hybridized to (*A*) ^{32}P-labeled 1.7-kb *ACT1 Bam*HI–*Hin*dIII fragment and 2.2-kb *CRY1 Hin*dIII fragment homologous to *CRY1* and *SNR189* RNA or (*B*) ^{32}P-labeled plasmid 11-10 encoding an abundant yeast mRNA and the 1.2-kb *RPL16A Bgl*II–*Bam*HI fragment homologous to *RPL16A* and *RPL16B* mRNA.

excess of mature *cry1* mRNA was present in the YEp24cry1 transformants, no unspliced *cry1* pre-mRNA accumulated. The *RPL16* genes do not contain introns.

Excess mRNAs are translated at wild-type rates

Two methods were used to determine whether excess *cry1* or *RPL16* mRNA was translated at wild-type rates. We measured the amount and distribution of these mRNAs in polyribosomes and we directly measured the rate of synthesis of these proteins by pulse labeling. If excess mRNA is translated, it will be associated with polyribosomes. A reduction in the rate of initiation or elongation of translation of these RNAs will result in the association of the mRNAs with smaller or larger than normal size classes of polyribosomes, respectively (Baim et al. 1985). Extracts from cells transformed with YEp24, YEp24cry1, and YEp24L16A were fractionated by centrifugation on 7% to 47% sucrose gradients that resolve polyribosomes, ribosomes, and postribosomal molecules. RNA extracted from each gradient fraction was subjected to gel electrophoresis, transferred to a Nytran filter, and hybridized with *CRY1*, *RPL16A* and *ACT1* probes. Most (>95%) of the excess *cry1* and *RPL16A* mRNA was found associated with monosomes and polyribosomes (Fig. 3). Furthermore, the size distribution of polyribosomes translating these excess *cry1* or *RPL16* mRNAs relative to those translating *ACT1* mRNA, was identical to that in YEp24 vector-transformed cells containing wild-type levels of the mRNAs. Less than 5% of the *cry1* or *RPL16A* mRNA was detected at positions in the gradient where free mRNA or mRNPs would sediment.

The relative rates of synthesis of rp59 or L16 proteins were measured in yeast transformed with YEp24, YEp24L16A, or YEp24cry1 by pulse labeling these cells for 30 sec with [^{35}S]methionine, mixing them with cells labeled for two generations with [^{3}H]methionine, extracting proteins and subjecting them to electrophoresis in a two-dimensional gel system (Gorenstein and Warner 1976) capable of resolving most ribosomal proteins (Fig. 4A). Synthesis of *rp59* or *L16* was quantitated by autoradiography or direct counting of incorporated radioactivity. The intensity of the rp59 spot relative to other ribosomal proteins was clearly increased in cells containing YEp24cry1 (Fig. 4B). Similarly, the amount of radioactivity incorporated into L16 protein was increased relative to all other ribosomal proteins in cells transformed with YEp24L16A (Fig. 4C). In separate experiments, spots containing different ribosomal proteins were excised from the gels and counted by liquid scintillation. The relative rate of synthesis of each ribosomal protein in cells containing elevated gene dosage is expressed as A_i (recombinant transformant)/A_i (vector transformant), where A_i is the ratio of ^{35}S/^{3}H cpm for that ribosomal protein divided by the average value of ^{35}S/^{3}H cpm for all other ribosomal proteins assayed (Table 1). By these criteria, the rates of synthesis of rp59 and L16 were increased roughly in proportion to the amount of excess *cry1* or *RPL16A* mRNA present (Table 1). The relative rates of synthesis of 30 to 50 other ribosomal proteins were unchanged in cells producing excess rp59 or L16 proteins.

Turnover of excess rp59 and L16 ribosomal proteins

We performed pulse-chase experiments to determine the fate of excess rp59 or L16 proteins. Cells were pulse labeled with [^{35}S]methionine for 30 sec, chased with excess cold methionine, and harvested with cells that were labeled for two generations with [^{3}H]methionine. Radioactivity present in ribosomal proteins at various times after the chase was determined by gel electrophoresis and scintillation counting. During the chase the relative A_i values of rp59 or L16 proteins decreased to a level close to those of other ribosomal proteins. The relative A_i values of rp59 and L16 reached a minimum after 14 min for rp59 and 40 min for L16 (Fig. 5). From semilogarithmic plots of A_i versus time, we derived that the excess rp59 and L16 proteins have apparent half-lives of 4 min and 24 min, respectively (data not shown).

Response to a rapid increase in RPL16A *mRNA levels*

In the elevated gene dosage experiments described above, the levels of mRNAs and rates of protein synthesis were measured many generations after the cells were transformed with extra copies of the genes, and may represent the result of long-term adaptation to ele-

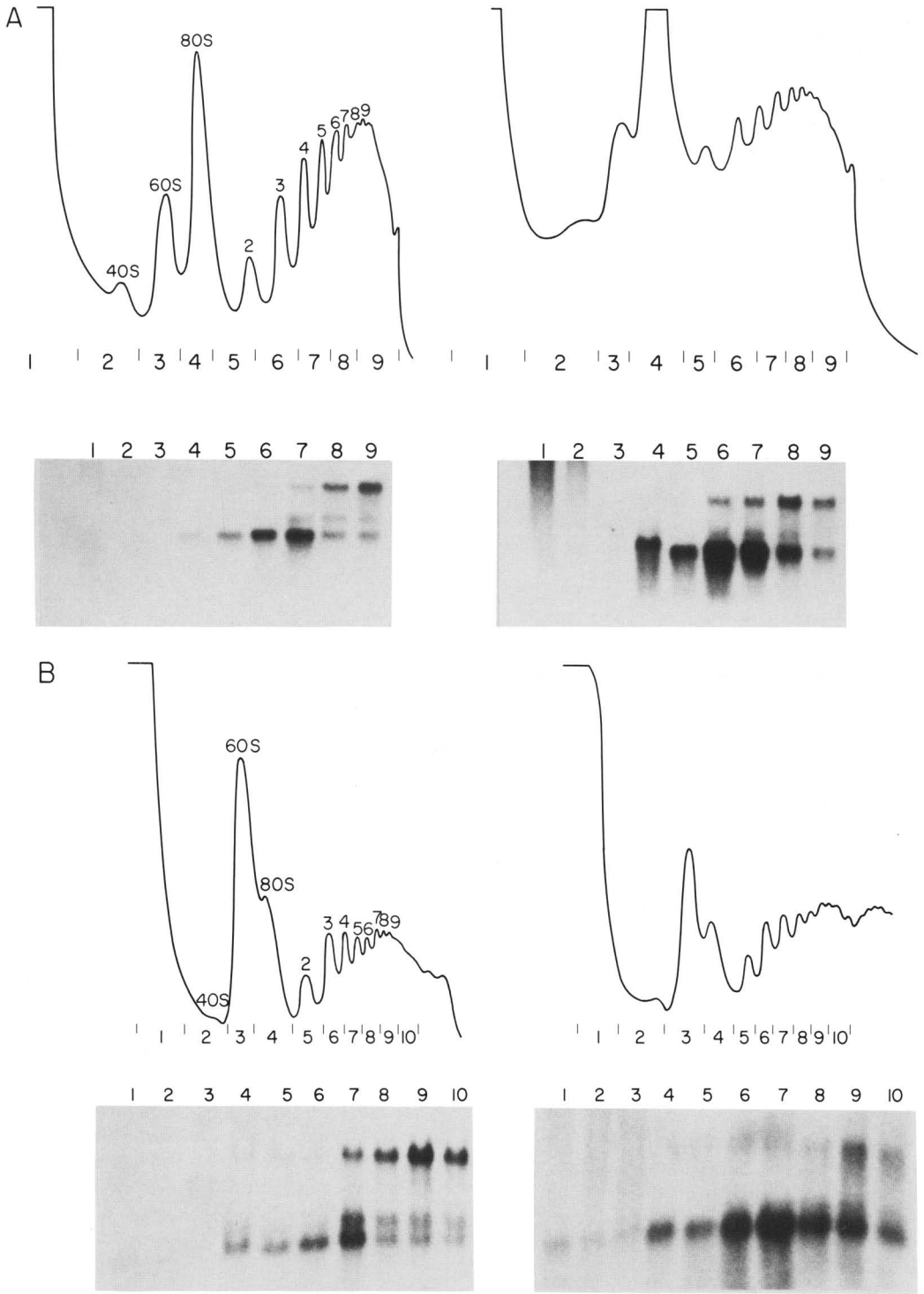

Figure 3. Distribution of excess *cry1* and *RPL16* mRNAs in monosomes and polyribosomes from cells containing elevated dosage of *cry1* or *RPL16*. RL78(*A*) or JL8(*B*) yeast transformed with YEp24 (*A* and *B*; *left*), YEp24cry1 (*A*; *right*), or YEp24L16A (*B*; *right*) were grown at 30°C in complete synthetic medium lacking uracil to a density of 10^8 cells/ml. The cells were harvested and their lysates were fractionated by centrifugation on 7% to 47% sucrose gradients to resolve polyribosomes, 80S monosomes, 40S and 60S ribosomal subunits, and postribosomal fractions. RNA was extracted from each fraction of the gradient and assayed by use of denaturing gel electrophoresis, transfered to Nytran filters, and hybridized with ^{32}P-labeled *ACT1*, *CRY1*, and *RPL16* probes. The *ACT1* probe, which also included the adjacent *YP2* gene, was used as an internal standard to control for variations in centrifugation or fractionation of polyribosomes. The smear in lanes *1* and *2* of (*A*; *right*) resulted from YEp24cry1 plasmid DNA at the top of the gradient.

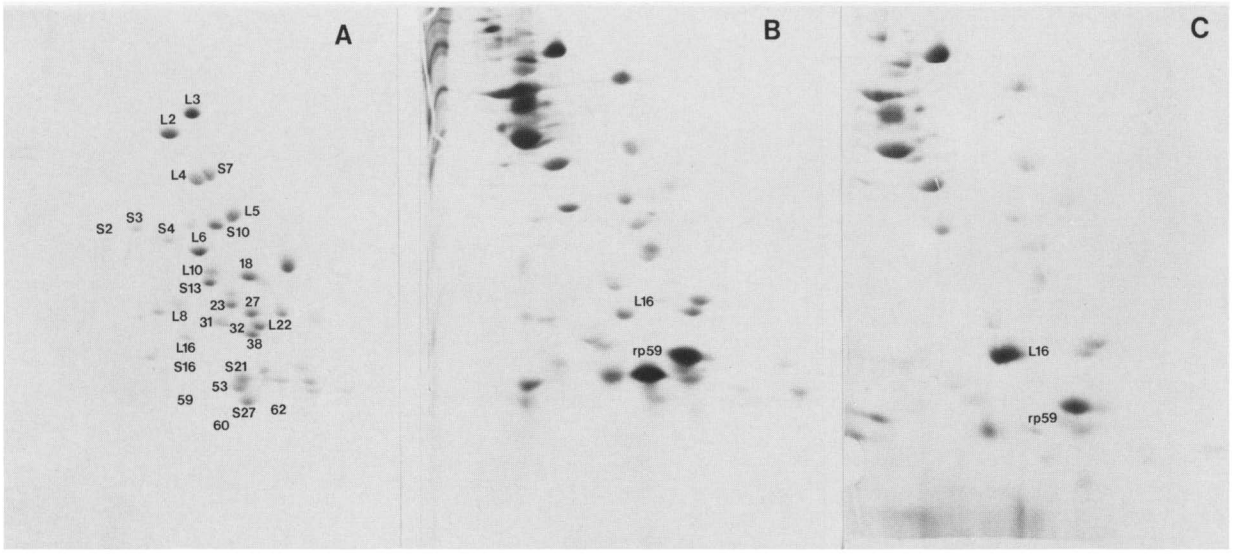

Figure 4. Synthesis of excess rp59 or L16 ribosomal proteins in yeast containing high-copy plasmids YEp24cry1 or YEp24L16A. (*A*) Coomassie brilliant blue-stained gel displaying yeast ribosomal proteins. The proteins are labeled according to the standard nomenclature (Warner 1982), using large (*L*) or small (*S*) subunit designations. In cases where the proteins have not been identified by this system, the nomenclature of Gorenstein and Warner (1976) is used. (*B* and *C*) RL78 yeast transformed with either (*B*) YEp24cry1 or (*C*) YEp24L16A were pulse labeled with 1 mCi of [^{35}S]methionine for 30 sec, and ribosomal proteins were extracted and subjected to electrophoresis in two dimensions. The fluorographs of the gels demonstrate a synthesis of excess rp59 and L16 proteins in the respective transformants.

vated synthesis of ribosomal protein mRNAs or ribosomal proteins. A more sensitive test of the cell's response to modulations in ribosomal protein gene or transcript levels would be to measure ribosomal protein synthesis immediately after excess mRNA is synthesized. Therefore, we constructed a yeast strain in which the transcription of *RPL16A* could be rapidly increased in response to a particular signal. The promoter of the *RPL16A* gene was replaced with that from the inducible *GAL1* gene of yeast (Johnston and Davis 1984). The *GAL1* promoter is approximately a thousand-fold more active in medium containing galactose than that containing glycerol and lactate (St. John and Davis 1981). This *GAL1–RPL16A* promoter fusion (Fig. 6) was inserted into a yeast low-copy centromere vector to create plasmid pBM1-35, and was transformed into strain SC252. In some experiments, the steady-state level of *RPL16* mRNA increased as much as eight-fold within 40 min after shifting cells containing the *GAL1–RPL16A* gene from a medium containing 3% glycerol and 2% lactate to a 2% galactose carbon source (Fig. 7). The cells were pulse labeled for 30 sec at 15 min after the carbon source shift, when the rate of increase of *RPL16* mRNA was most rapid, or at 24 min, just before *RPL16* mRNA levels reached a maximum level (Fig. 7). At both times the rate of synthesis of L16 protein was elevated in proportion to the amount of excess *RPL16* mRNA present (Table 2). Pulse-chase experiments demonstrated that the excess L16 protein synthesized at these times decayed with kinetics similar to those measured in cells transformed with high copies of *RPL16A* (data not shown).

Table 1. *Synthesis of excess* cry1 *and* RPL16 *mRNA and proteins in cells containing elevated dosage of* cry1 *or* RPL16 *genes*

Plasmid		Relative mRNA levels	Relative ribosomal protein synthesis rate
YEp24cry1	expt. 1	7.8	7.7
	expt. 2	10.5	10.4
YEp24L16A	expt. 1	5.5	4.3
	expt. 2	14.4	9.7

Transformants of yeast strain RL78 were grown to mid-log phase in synthetic medium selecting for retention of the plasmid. RNA was extracted from aliquots of each culture to determine mRNA abundance. *cry1*, *RPL16*, and *ACT1* mRNA levels were measured by scintillation counting of mRNA bands excised from an RNA gel blot. The values indicated are ribosomal protein mRNA levels normalized to *ACT1* mRNA in the same gel lane, and are compared to those from vector transformed cells. Other aliquots of cells were pulse labeled with [^{35}S]methionine and ribosomal proteins were extracted and separated by two-dimensional gel electrophoresis. The relative rates of synthesis for the individual proteins were determined by scintillation counting. The relative rates of synthesis for 30 other ribosomal proteins were identical in vector and recombinant transformants with an average A_i value of 1.0 ± 0.25 S.D.

Replacement of the 5′ noncoding sequences of RPL16 *has no effect on translation of* RPL16A *mRNA or turnover of L16 protein*

In the experiments described above, the measured rate of synthesis of excess L16 protein was consistently from 20 to 30% less than the total amount of *RPL16A* mRNA. Therefore, the possibility remained that some transla-

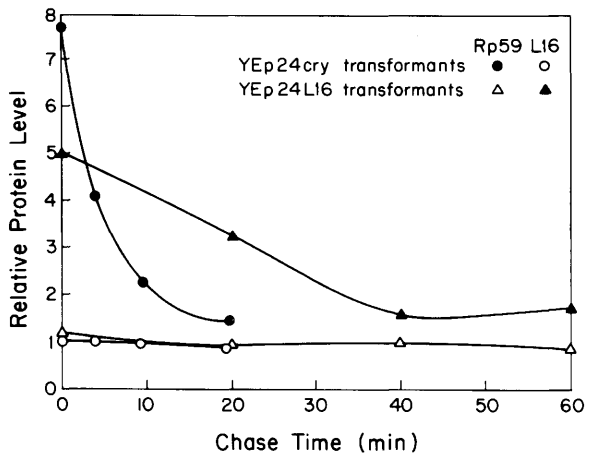

Figure 5. Turnover of excess rp59 or L16 ribosomal proteins synthesized in yeast containing an elevated number of *cry1* or *RPL16* genes. RL78 yeast transformed with YEp24*cry1* (○ ●), or YEp24L16A (△ ▲) were pulse-labeled for 30 sec with 1 mCi of [^{35}S]methionine followed by addition of unlabeled methionine to 268 mM. At the indicated times, cells were harvested, and ribosomal proteins were extracted and assayed by two-dimensional gel electrophoresis and scintillation counting of spots containing rp59 or L16. The relative levels of rp59 or L16 are expressed as a ratio of A_i values for each protein in cells containing elevated dosage of that gene compared to those containing wild-type gene dosage. Closed symbols indicate protein synthesized in cells containing elevated dosage of that gene, and open symbols represent protein in cells containing wild-type gene dosage.

tional regulation of *RPL16A* expression might occur. We performed the following experiment to determine whether or not expression of *RPL16A* is mediated, even in part, by a mechanism of translational repression utilizing the 5′ noncoding region of *RPL16A* transcripts.

We constructed a *GAL1–RPL16A* gene fusion in which the entire *RPL16A* 5′ nontranslated leader up to the AUG initiation codon was replaced by the *GAL1* 5′ leader sequence (Fig. 6). This hybrid gene also contains the *GAL1* promoter in place of the *RPL16A* promoter. Therefore, the transcription of this hybrid *GAL1–RPL16A* gene is inducible by galactose. This *GAL1–RPL16A* gene fusion was cloned into a low-copy centromere plasmid to create plasmid pBM2-57 which was transformed into yeast. As was the case with the *GAL1–RPL16A* promoter fusion, *GAL1–RPL16A* mRNA levels increased three- to eightfold within 40 min after shifting these cells from medium containing 3% glycerol and 2% lactate to that containing 2% galactose (Table 2 and data not shown). The relative rate of synthesis and half-life of L16 protein at 15 min and 24 min after the carbon source shift was measured by pulse-labeling and pulse-chase experiments. The results obtained were identical to those found with the *GAL1–RPL16* promoter fusion; a two- to threefold excess of L16 was synthesized and decayed to levels identical to other ribosomal proteins (Table 2).

Turnover of excess L16 or rp59 protein is not mediated by vacuolar proteases

We tested whether vacuolar hydrolases are necessary for turnover of excess rp59 or L16 protein by examining the accumulation of these ribosomal proteins in cells containing null alleles of the *PEP4* and *PRB1* genes encoding vacuolar hydrolases A and B, respectively (Woolford et al. 1986; Moehle et al. 1987). These proteases are necessary for the activation of the zymogen forms of all known soluble vacuolar hydrolases (Woolford et al. 1986). A *pep4 :: HIS3 prb1-Δ1.6R* strain and an otherwise isogenic wild-type strain were transformed with

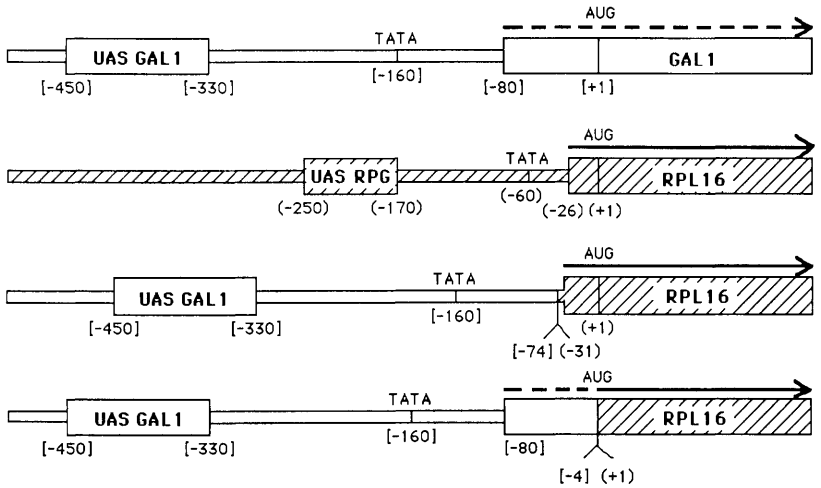

Figure 6. Replacement of the *RPL16A* promoter and 5′-noncoding sequences with those of the yeast *GAL1* gene. (*Top*) The wild-type *GAL1* gene. The locations of the upstream activating sequences (UAS) or promoter, the TATA consensus sequence, the *GAL1* mRNA, and the *GAL1* translation initiation codon AUG are shown. (*Second from top*) The wild-type *RPL16A* gene, labeled as in *A*. (*Third from top*) *GAL1–RPL16* fusion in plasmid pBM1-35. The *RPL16A* promoter was replaced by the *GAL1* promoter, so that the *RPL16A* transcription initiation site is placed at a distance from the *GAL1* promoter that is approximately equal to the distance naturally occurring between the *GAL1* transcription initiation site and *GAL1* promoter. The *RPL16A* gene, extending from nucleotide −31 with respect to the translation initiation codon AUG, i.e., 5 nucleotides upstream of the *RPL16A* transcription initiation site, was inserted downstream from the yeast *GAL1* promoter in plasmid pBM126, at nucleotide −74. This position is 12 nucleotides 5′ of the *GAL1* transcription initiation site in the wild-type *GAL1* gene. (*Bottom*) *GAL1–RPL16* fusion in which the *RPL16A* promoter and entire 5′-noncoding sequence is replaced with the *GAL1* promoter and almost all of the *GAL1* 5′ leader sequence. The *RPL16A* gene, extending from nucleotide +1 (the A of the AUG initiation codon) was inserted in plasmid pBM258 downstream of the *GAL1* promoter and 5′-noncoding sequences at nucleotide −4 with respect to the *GAL1* AUG translation initiation codon.

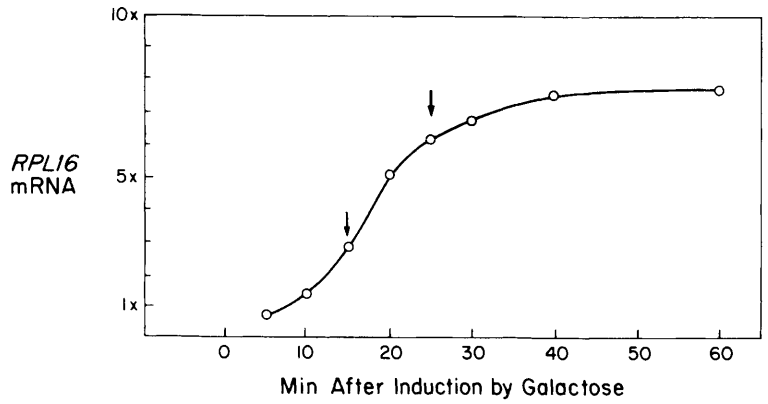

Figure 7. Time course of accumulation of *RPL16* mRNA expressed from a *GAL1–RPL16A* promoter fusion. Yeast strain SC252 was transformed with plasmid pBM1-35 containing the *RPL16A* gene under control of the *GAL1* promoter. At the indicated times (↓) after shifting the cells from the glycerol and lactate medium to the galactose-containing medium, RNA was extracted from the cells and assayed by RNA gel transfer hybridization for the amount of *RPL16* mRNA relative to *ACT1* mRNA. The ratios of *RPL16* mRNA to *ACT1* mRNA (determined by scintillation counting) are normalized relative to those present in cells growing in glycerol-lactate medium prior to the carbon source shift.

Table 2. *Synthesis and turnover of excess ribosomal protein L16 expressed from* GAL1–RPL16A *promoter fusion and 5′ leader replacement constructs*

Plasmid	Minutes after carbon source shift	Relative excess of L16 mRNA	Relative synthesis of L16
Vector control (pBM258)	15	1	1.0
	24	1	1.1
Promoter replacement (pBM1-35)	15	2.6	1.8
	24	3.5	3.2
Promoter and 5′ nontranslated leader replacement (pBM2-57)	15	3.2	2.1
	24	4.8	3.3

Transformants of yeast strain SC252 were shifted from a glycerol and lactate medium to galactose medium in order to induce the *GAL1* promoter. At the indicated times after shift, the relative rates of L16 protein synthesis were determined by a 30-sec pulse with [^{35}S]methionine. The mRNA levels were determined by gel transfer hybridization.

Table 3. *Identical turnover of excess rp59 and L16 proteins in wild-type yeast and strains deficient in vacuolar hydrolases*

Host strain	Plasmid	Relative synthesis rates of L16 or rp59	
		pulse	chase
Wild-type	YEp24cry1	3.0	1.5
Protease deficient	YEp24cry1	6.2	1.6
Wild-type	YEp24L16B	5.2	2.6
Protease deficient	YEp24L16B	4.2	2.0

Logarithmically growing cells were pulse labeled for 30 sec with [^{35}S]methionine and chased with excess cold methionine for at least one half-life (length of chase was 30 min for L16, 7 min for rp59). Relative synthesis rates were determined as described in Materials and methods. The protease deficient strain used was BJ3501 (*MATα pep4::HIS3 prb1–Δ1.6R ura3–52 his3Δ200 can1* Gal$^+$). This strain lacks the zymogen-activating proteases A and B and is therefore deficient in all known vacuolar hydrolase activities. The wild-type strain used was YP102 (*MATα PEP4 PRB1 ura3–52 his3Δ200 leu2Δ1 lys2–801 ade2–101*).

YEp24cry1 and YEp24L16B. The rate of synthesis and turnover of rp59 and L16 measured by pulse-labeling and pulse-chase experiments were very similar in these wild-type and protease deficient strains (Table 3). We conclude that vacuolar hydrolases are not directly responsible for the turnover or the modification of ribosomal proteins that results in their disappearance from their normal position in a two-dimensional gel.

Discussion

Accumulation of equimolar quantities of ribosomal proteins during steady-state growth probably does not result simply from transcription of each ribosomal protein gene at equivalent rates into mRNAs that are translated with equal efficiency. It is more likely that modest differences in rates of transcription of the different ribosomal protein genes are compensated by a combination of posttranscriptional mechanisms. Modulation of translation of ribosomal protein genes has been shown to be responsible for changes in ribosome synthesis during development or in response to external stimuli (Kief and Warner 1981; Kim and Warner 1983a; Baum and Wormington 1985; Kay and Jacobs-Lorena 1985; Pierandrei-Amaldi et al. 1985; Schmidt et al. 1985; Donovan and Pearson 1986; Herruer et al. 1987). An elegant model for the autogenous regulation of translation of *E. coli* ribosomal protein operons by a mechanism coupled to ribosome assembly has been developed to explain balanced synthesis of ribosomes during steady-state growth (reviewed in Nomura et al. 1984). Originally several investigators observed a large discrepancy between the elevated dosage of eukaryotic ribosomal protein genes or mRNAs, and the apparent rate of ribosomal protein synthesis. This led them to conclude that balanced synthesis of eukaryotic ribosomal proteins during steady-state growth resulted either from repression of translation of excess mRNA or from turnover of excess protein, but these two possibilities could not be distinguished (Pearson et al. 1982; Himmelfarb et al. 1984; Warner et al. 1985).

Our experiments and those of others suggest that the predominant form of posttranscriptional regulation of ribosomal protein production during steady-state growth

of yeast and other eukaryotic cells occurs at the level of turnover of excess ribosomal proteins rather than translational control (Table 4). Increasing the dosage of ribosomal protein genes in most cases leads to synthesis of excess mRNAs and proteins (Abovich et al. 1985; Warner et al. 1985; El Baradi et al. 1986; Bowman 1987; Baum et al. 1988; Maicas et al. 1988; Pierandrai-Amaldi et al. 1988; M. Jacobs-Lorena, pers. comm.). The use of very short pulse labeling times, rapid extraction protocols, and in some cases pulse-chase analysis established that excess ribosomal proteins are made and rapidly degraded under these conditions. We found no evidence for regulation of splicing of the intron-containing *cry1* transcript or for modulation of stability of excess *RPL16* or *cry1* mRNAs.

These gene dosage experiments have provided no compelling evidence for translational control under these experimental conditions. Nevertheless, the formal possibility remained that translational control could be a minor component of the regulatory response of ribosomal proteins as suggested by the small discrepancy between the levels of excess mRNA and ribosomal protein synthesis. Translational control in eukaryotes is often mediated by sequences in the 5′ noncoding region of mRNAs (McGarry and Lindquist 1985; Pelletier and Sonenberg 1985; Costanzo and Fox 1986; Mueller and Hinnebusch 1986). Because replacement of the *RPL16A* promoter and 5′ leader sequence with that of another yeast gene, *GAL1*, did not affect the posttranscriptional expression of *RPL16A*, we conclude that it is unlikely that expression of *RPL16A* is balanced with that of other yeast ribosomal protein genes by regulation of its translation. We presume that the small differences in levels of ribosomal protein mRNA and ribosomal protein synthesis that we do observe result from degradation of ribosomal proteins during extraction of labeled cells.

Analysis of unbalanced overproduction of a single ribosomal protein, such as in our increased gene dosage experiments, examines the cell's long-term adaptive response to a steady-state overproduction of a ribosomal protein. We determined as well that the response of ribosome biosynthesis to a sudden increase in ribosomal protein mRNA was identical to that in steady-state experiments that used high-copy number transformants.

Turnover of excess ribosomal components occurs under a variety of other circumstances. When ribosomal protein synthesis is inhibited, rRNA precursors are synthesized, but are not processed and are degraded (Shulman and Warner 1978; Stoyanova and Hadjiolov 1979). In the absence of ribosomal RNA synthesis , ribosomal proteins are synthesized at normal rates but degraded. This occurs in anucleolate *Xenopus* (Pierandrei-

Table 4. *Summary of ribosomal protein gene dosage experiments in eukaryotes*

	Regulation of			
Ribosomal protein gene	pre-mRNA splicing[a]	translation[b]	protein turnover[c]	reference
Yeast *RP51*	–	–	+	Abovich et al. (1985)
Yeast *RPS7*	n.a.	–	+	Warner et al. (1985)
Yeast *RPS10*	–	–	+[d]	Warner et al. (1985)
Yeast *RP29*	–	–	+	Warner et al. (1985)
Yeast *RPL32*	+	n.d.	n.d.	Warner et al. (1985)
Yeast *RPL25*	–	–	+	El Baradi et al. (1985)
Yeast *RPL3* (*TCM1*)	n.a.	–	+	Maicas et al. (1988)
Yeast *RPL29* (*CYH2*)	+	–	+	Maicas et al. (1988)
Yeast *RP59* (*CRY1*)	–	–	+[d]	Maicas et al. (1988)
				this study
Yeast *RPL16*	n.a.	–	+[d]	this study
Drosophila rp49	–	–	+	M. Jacobs-Lorena, pers. comm.
Xenopus L1	+	–	+	Baum et al. (1988)
				Bozzoni et al. (1984)
				Pierandrei-Amaldi et al. (1988)
Xenopus L14	–	–	+	Baum et al. (1988)
				Bozzoni et al. (1984)
				Pierandrei-Amaldi et al. (1988)
Mouse *S16*	–	–	+	Bowman (1987)
Mouse *L32*	–	–	+[d]	Bowman (1987)

Elevated mRNA levels were achieved by increased gene dosages in yeast, *Drosophila*, mouse myoblast cell lines, and *Xenopus* oocytes, or by injection of excess RNA into *Xenopus* oocytes.

[a] Regulation of splicing was manifest by accumulation of excess unspliced pre-mRNA.

[b] Translational control was assayed by measurement of the distribution of excess mRNA in polyribosomes or the synthesis of excess protein.

[c] Turnover was inferred by an increase in the apparent synthetic rate with increasingly shorter pulse-labeling periods.

[d] In these cases, turnover of excess protein was verified by pulse-chase analysis.

n.a. Not applicable; these genes contain no introns.

n.d. Not determined; the existence of translational control or turnover could not be determined since no excess mature mRNA was present in these cells.

Amaldi et al. 1985), *bobbed* mutants of *Drosophila* (Kay and Jacobs-Lorena 1985), differentiating rat myoblasts (Jacobs et al. 1985), in the yeast rRNA processing mutant ts351 (Gorenstein and Warner 1977), or upon inhibition of rRNA synthesis by actinomycin D in rat liver or HeLa cells (Craig and Perry 1971; Tsurugi and Ogata 1977; Warner 1977). Furthermore, ribosomal proteins injected into *Xenopus* oocytes are unstable if they do not assemble into ribosomes (Kalthoff and Richter 1982; Baum 1986). When expression of a single yeast ribosomal protein is decreased or inhibited, other ribosomal proteins and rRNA of the corresponding ribosomal subunit are synthesized but degraded (Abovich et al. 1985; Nam and Fried 1986). On the other hand, during embryogenesis of *Drosophila* or *Xenopus*, a few exceptional ribosomal proteins are synthesized and remain stable in the absence of rRNA synthesis and ribosome assembly (Pierandrei-Amaldi et al. 1982; Kay and Jacobs-Lorena 1985).

Excess ribosomal proteins may be identified for turnover simply by their inability to assemble into ribosomes. Such a process would most likely be initiated in the nucleolus where ribosomes assemble. Alternatively, turnover could occur in the cytoplasm, either upon failure of ribosomal proteins or subassembly complexes of ribosomal proteins to enter the nucleolus, or upon their exit from the nucleolus in the absence of assembly. Turnover of ribosomal proteins in our pulse-chase experiments is functionally defined as a decrease in the levels of a radioactive protein at its characteristic position in a two-dimensional gel system. The apparently rapid degradation of excess protein that we observed may result from a single proteolytic cleavage or a covalent modification that is only the first step in a degradative pathway.

The half-lives of excess rp59 and L16 proteins are very different, 4 min and 24 min, respectively. These values fall within the observed range of half-lives of yeast ribosomal proteins that are degraded upon inhibition of rRNA splicing in the ts351 mutant (Gorenstein and Warner 1977). Under these circumstances, the half-life of L16 was 24 min; that of rp59 was not measured. The differences in half-lives between ribosomal proteins may simply be a function of the structure of the unassembled proteins or may reflect the mechanism by which each protein assembles into ribosomes. We have begun to investigate whether a mechanism specific to ribosome assembly or more general mechanism is used in the turnover of excess ribosomal proteins.

Materials and methods

Nomenclature of ribosomal protein genes

The duplicated yeast genes encoding the 60S ribosomal subunit protein L16 are designated *RPL16A* and *RPL16B* (Rotenberg et al. 1988), based on standard nomenclature of yeast ribosomal proteins (Warner 1982). We had previously denoted this protein rp39 and its genes *RP39A* and *RP39B* (Woolford et al. 1979; Teem et al. 1984; Rotenberg and Woolford 1986), using the original nomenclature of Gorenstein and Warner (1976). The 40S ribosomal subunit protein rp59 is encoded by two genes denoted *CRY1* and *CRY2* (Larkin and Woolford 1983; A.G. Paulovich et al. in prep.). The wild-type alleles *CRY1* and *CRY2* confer sensitivity to cryptopleurine, an inhibitor of the EF-2, GTP-dependent step of translocation in protein synthesis (Skogerson et al. 1973; Grant et al. 1974; Dolz et al. 1982). The recessive *cry1* allele is responsible for conferring resistance to cryptopleurine. The *CRY* gene products have been designated rp59 in the gel system that we use (Gorenstein and Warner 1976); the counterpart for rp59 has not been identified in the standard two dimensional gel system (Warner 1982).

Materials and enzymes

T4 DNA ligase, [α-^{32}P]dCTP, and [methyl-^{3}H]L-methionine (200 Ci/mmole) were purchased from New England Nuclear Corp. Tran ^{35}S-label (L-methionine, [^{35}S]; L-cysteine, [^{35}S], >1000 Ci/mmole, and [methyl-^{3}H]L-methionine (5–15 Ci/mmole) were purchased from ICN Biomedicals, Inc. [α-^{32}P]dATP (>400 Ci/mmole) was obtained from Amersham Corp. Restriction endonucleases, nuclease *BAL31*, and calf alkaline phosphatase were purchased from Boehringer-Mannheim Biochemicals. *Bam*HI and *Bgl*II linkers and certain restriction endonucleases were obtained from New England Biolabs. *E. coli* DNA polymerase I holoenzyme and Klenow fragment were the kind gift of Dr. William E. Brown, Carnegie Mellon University. DEAE-nitrocellulose (NA45), nitrocellulose (0.45 μM), and Nytran (0.45 μM) were purchased from Schleicher and Schuell. Sodium dodecyl sulfate used in two-dimensional polyacrylamide gels was obtained from Pierce Chemical Company.

Plasmids, strains, and media

The parent plasmid used for high-copy number transformation was YEp24 (Botstein et al. 1979). YEp24cry1 contains the 5.0-kb *Bam*HI–*Sal*I fragment bearing the entire *cry1* allele and the *SNR189* gene encoding a small nuclear RNA, inserted between the *Bam*HI and *Sal*I sites of YEp24. Plasmid YEp24L16A containing the *RPL16A* gene was obtained by screening a library of yeast genomic DNA, cloned as partial *Sau*3A fragments in YEp24 (Carlson and Botstein 1982), using the ^{32}P-labeled 1.2-kb *Bgl*II–*Bam*HI *RPL16A* fragment as a colony hybridization probe. Plasmid YEp24L16B was constructed by inserting the 3.0-kb *RPL16B Hin*dIII–*Bam*HI fragment isolated from plasmid pY11-40HB (Rotenberg et al. 1988), into the *Bam*HI site of YEp24. This *Hin*dIII–*Bam*HI fragment was converted to a *Bam*HI fragment by filling in the 5′ ends using Klenow fragment of DNA polymerase, ligating *Bam*HI linkers, and removing excess linkers by digestion with *Bam*HI, as described in Rotenberg and Woolford (1986). A 2.2-kb *Eco*RI–*Hin*dIII fragment from plasmid pYACT1 (Gallwitz and Seidel 1980) was used as a hybridization probe for *ACT1* and *YP2* mRNAs.

A collection of 5′ deletion derivatives of the *RPL16A* gene, extending from 144 base pairs upstream of the transcription initiation site of the gene into the 5′ noncoding region, was constructed and inserted downstream of either the *GAL1* promoter in plasmid pBM126 or the *GAL1* promoter and most of the *GAL1* 5′ noncoding region in plasmid pBM258 (Johnston and Davis 1984). The 1.2-kb *Bgl*II–*Bam*HI fragment containing *RPL16A* was isolated from plasmid pY-1078 (Woolford et al. 1979) and cloned into the *Bam*HI site of plasmid pUC8, such that the *Sma*I site in the pUC8 polylinker was 5′ of the *Bgl*II end of the fragment (5′ end of *RPL16A*) and the *Hin*dIII site in the polylinker was 3′ of the *Bam*HI end of the fragment. This plasmid was linearized by digestion with *Sma*I; deletions extending from the *Sma*I site were made by incubation with 0.2 U *BAL*31 nuclease per 25 μg of DNA, as described in Rotenberg and Woolford (1986). Reactions were terminated at different

times by adding chilled EGTA to 20 mM. The ends of the BAL31 treated DNA were filled in by treatment with DNA polymerase I Klenow fragment, *Bgl*II restriction enzyme site linkers were ligated to the ends, and excess linkers were removed by digestion with *Bgl*II. The resulting DNA was recircularized by ligation using T4 DNA ligase, and was transformed into *E. coli*. The sizes of the deletions within various plasmid DNAs were estimated by analysis of the pattern of the *Hin*fI or *Hae*III restriction enzyme fragments derived from them. The *Bgl*II–*Bam*HI fragments in the appropriate size range were cloned into the *Bam*HI site of pBM126 and pBM258, downstream of the *GAL1* promoter. These two vectors contain the *GAL1–GAL10* intergenic region including the promoters of these two genes and the yeast *CEN4*, *ARS1*, and *URA3* sequences (Johnston and Davis 1984). Two constructs, pBM1-35 derived from pBM126, and pBM2-57 derived from pBM2-58, were chosen for further use. The nucleotide sequence of the *GAL1–RPL16* junction was determined for each construct. pBM1-35 contains the *GAL1* promoter and most of the *GAL1* 5′ nontranscribed region fused (via a linker) to the 5th nucleotide upstream of the *RPL16A* transcription initiation site (Fig. 6). pBM2-57 contains the *GAL1* promoter, 5′ nontranscribed region, and most of the *GAL1* 5′ transcribed, nontranslated sequences fused via a linker to the *RPL16A* AUG translation initiation codon.

Yeast strains used in this study were RL78 (*MATa ura3-52*), JL8 (*MATα cry1 leu2-3,112 trp1 ura3-52*), YP102 (*MATα ade2-101 his3-Δ200 leu2-Δ1 lys2-801 ura3-52*), BJ3501 (*MATα can1 Gal⁺ his3-Δ200 pep4 :: HIS3 prb1-Δ1.6R ura3-52*), and SC252 (*MATα ade1 leu2-3,112 ura3-52 Gal⁺*). RL78 and JL8 were constructed in this laboratory (Last et al. 1984; Larkin et al. 1987), YP102 was obtained from Phil Hieter, and SC252 from Jim Hopper. BJ3501 was derived in Elizabeth Jones' laboratory by crossing a strain containing a null allele of *pep4*, *pep4 :: HIS3*, (constructed by M. Innis, Cetus Corp.) with one containing a *prb1* null allele, *prb1-Δ1.6R* (Moehle et al. 1987). *E. coli* strains HB101, JA221 (Clarke and Carbon 1978), or RR1 (Maniatis et al. 1982) were used as bacterial hosts for all plasmid DNAs. Yeast were grown either in YEPD (1% yeast extract, 2% peptone, 2% dextrose) or synthetic medium; bacterial cells were grown in LB medium (5 grams NaCl, 5 grams yeast extract, 10 grams Tryptone per liter) supplemented with ampicillin as described previously (Larkin et al. 1987).

Transformation

Plasmid DNAs were transformed into *E. coli* HB101, JA221, or RR1 with selection for ampicillin resistance as described by Davis et al. (1980). Yeast were transformed with plasmid DNAs by the spheroplast method as described by Sherman et al. (1979), the lithium acetate method described by Ito et al. (1983), or by a modification of the lithium acetate method using calcium chloride, as described by Woolford et al. (1986).

Isolation, electrophoresis, and gel transfer analysis of nucleic acids

Plasmid DNAs, yeast genomic DNA, DNA restriction fragments, and yeast RNAs were prepared as described previously (Last et al. 1984; Larkin et al. 1987). Restriction endonuclease digestions, BAL31 nuclease digestions, filling-in of 5′ sticky ends of DNA with DNA polymerase Klenow fragment, and DNA ligations were performed as described in Last et al. (1984) or Rotenberg and Woolford (1986). Electrophoresis of DNA on 1.0% agarose gels and of RNA on 1.2% agarose–formaldehyde gels, transfer to nitrocellulose or Nytran filters, and hybridization to in vitro-labeled DNA probes were performed as described (Last et al. 1984; Larkin et al. 1987). DNAs used as hybridization probes were labeled with ^{32}P in vitro by nick translation (Rigby et al. 1977) or by the random primed synthesis protocol (Feinberg and Vogelstein 1983). Densitometry of autoradiograms was performed by scanning several exposures of each blot using the tungsten lamp of a Zeineh soft laser scanning densitometer (model SL-504-XL).

Measuring rates of synthesis of ribosomal proteins

The relative rates of synthesis of ribosomal proteins were measured by pulse labeling as follows: Yeast cells transformed with plasmids containing the yeast *URA3* gene were grown at 30°C in synthetic complete medium lacking uracil and methionine to an A_{610} of 0.1 to 0.2 units. [^{3}H]methionine (80 μCi) was added to 1 ml of these cells, which were grown for an additional two generations (5 hr). Another aliquot of the same cells was grown to an A_{610} of 0.5 to 0.6 units. [^{35}S]methionine (700–1000 μCi) was added to 6 ml of these cells. The ^{35}S-labeled cells were harvested 30 sec later by mixing them with the ^{3}H-labeled cells and vacuum filtering them through a 0.45 μm Metricel membrane filter (Gelman Scientific, Inc., Ann Arbor, Michigan). The cells were quickly washed on the filter with 10 ml of ice cold water and immediately frozen by laying the filter in a glass petri dish on top of a block of dry ice. The frozen cells were broken open by mixing them in a 15 ml Corex tube with 0.5 ml of glass beads and 1 ml of ice cold lysis/extraction buffer (30 mM $MgCl_2$, 3 mM DTT, 1.8 mM PMSF, 70% glacial acetic acid), and vortexing eight times for 30 sec each time with 30 sec intervals on ice between each vortexing. Lysis/extraction buffer (2.5 ml) was added to the extract which was incubated on ice for 30 min, and centrifuged at 16,000*g* for 10 min in an HB4 rotor. Ribosomal proteins were precipitated from the supernatant by addition of 5 volumes of acetone and incubation at −20°C overnight. The proteins were resolved by two-dimensional electrophoresis (Gorenstein and Warner 1976). Gels were subjected to fluorography or spots corresponding to ribosomal proteins were excised from the gel and radioactivity was measured by liquid scintillation counting, as described by Woolford et al. (1979) and Warner and Gorenstein (1978).

Identical means were used to assay rates of synthesis of ribosomal proteins after shifting cells from a glycerol and lactate medium to a galactose medium. Cells were grown in synthetic complete medium lacking uracil and methionine and containing 3% glycerol plus 2% lactic acid as a carbon source to an A_{610} of 0.07 units. [^{3}H]methionine was added to an aliquot of the cells which were grown for another 10 hr (two generations). The remainder of the culture was grown to A_{610} = 0.3 units, the cells were spun down, washed, and suspended in synthetic complete medium lacking uracil and methionine and containing 3% galactose. These cells were pulse labeled with [^{35}S]methionine for 30 sec, harvested together with the [^{3}H]-labeled cells, and ribosomal proteins were extracted and assayed as above.

Pulse-chase experiments were performed by labeling cells for 30 sec with [^{35}S]methionine, as described above, followed by addition of nonradioactive methionine to the medium to a final concentration of 268 mM. At various times thereafter, cells were harvested and processed as described above.

Distribution of mRNAs among postribosomal fraction, free ribosomal subunits, monosomes, and polyribosomes

Polyribosomes were isolated from yeast and fractionated as described by Rotenberg et al. (1988). The distribution and quantities of mRNAs in the gradient fractions were determined as

follows. RNA in each fraction was precipitated by addition of two volumes of ice cold ethanol, incubation at −20°C overnight, and centrifugation for 5 min at 16,000*g* in an HB4 rotor. The pellets were dissolved in 20 mM Tris–HCl (pH 8.0), 0.2 M NaCl, 3 mM EDTA, 0.1% diethyl pyrocarbonate, and 1% SDS; deproteinization was by extraction with phenol at 65°C, and the RNA in the supernatant was ethanol precipitated. Each RNA sample was subjected to electrophoresis, and quantitated by gel transfer to Nytran filters, hybridization, autoradiography, and scintillation counting of strips of the filters.

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